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# Purification and properties of a xylanase from *Streptomyces lividans*

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An extracellular xylanase produced by a cellulase-negative mutant strain of *Streptomyces lividans* 1326 was purified to homogeneity. The purified enzyme has an apparent $M_r$ of 43000 and pI of 5.2. The pH and temperature optima for the activity were 6.0 and 60 °C respectively, and the $K_m$ and $V_{max.}$ values, determined with a soluble oat spelts xylan, were 0.78 mg/ml and 0.85 mmol/min per mg of enzyme. The xylanase showed no activity towards CM-cellulose and *p*-nitrophenyl β-D-xyloside. The enzyme degraded xylan, producing mainly xylobiose, a mixture of xylo-oligosaccharides and a small amount of xylose as end products. Its pattern of action on β-1,4-D-xylan indicates that it is a β-1,4-endoxylanase (EC 3.2.1.8).

## INTRODUCTION

Hemicellulose is one of the major components of lignocellulosic biomass and consists largely of xylan. Xylanases play an important role in the decomposition of soil plant litter by micro-organisms. Microbial xylanases have been purified from *Aspergillus* (John *et al.*, 1979), *Trichoderma* (Baker *et al.*, 1977), *Schizophyllum* (Paice *et al.*, 1978), *Cryptococcus* (Morosoli *et al.*, 1986), *Bacillus* (Bernier *et al.*, 1983) and other genera (Woodward, 1984). The streptomycetes, too, are very active in the biochemical decomposition of lignocellulosic biomass in soils and have been reported to produce considerable amounts of this hemicellulase (Ishaque & Kluepfel, 1981). A xylanase from *Streptomyces* sp. E86 has been used for the production of xylobiose and xylose from commercial hardwood (Kusakabe *et al.*, 1975), and two xylanases were characterized from different *Streptomyces* species (Nakajima *et al.*, 1984; Ohwaki *et al.*, 1983). More recently we demonstrated the capacity of *Streptomyces lividans* cultures to excrete large amounts of xylanase (Kluepfel *et al.*, 1986), and in the present paper we describe the isolation and the purification of a β-1,4-endoxylanase from this micro-organism, as well as some of its properties.

## EXPERIMENTAL

### Preparative methods

**Organism and culture conditions.** *S. lividans* 1326 was kindly supplied by Dr. D. A. Hopwood (John Innes Institute, Norwich, U.K.). From this strain a β-1,4-glucan-glucanohydrolase-negative mutant (*S. lividans* 8-7) was obtained after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and selection for the absence of enzyme production by the method of Congo Red coloration (Wood, 1980). This mutant was used for all the experiments. It was maintained either freeze-dried or on 7-day-old slant cultures of tomato paste/oatmeal medium (Moldoveanu & Kluepfel, 1983). Spore suspensions, prepared from such slants with 10 ml of physiological saline (0.9% NaCl), were used to inoculate 100 ml of trypticase soy broth (TSB) into 500 ml Erlenmeyer flasks, which were incubated for 42 h on a rotary shaker (New Brunswick Sci. Co., model G25) at 250 rev./min at 34 °C. This culture served as inoculum for the following growth medium: xylan (from oat spelts; Sigma Chemical Co., St. Louis, MO, U.S.A.), 10 g; $(NH_4)_2SO_4$, 1.4 g; $K_2HPO_4$, 2.5 g; $KH_2PO_4$, 1.0 g; yeast extract (Difco), 2 g; proteose peptone (Difco), 1 g; $MgSO_4,7H_2O$, 0.3 g; $CaCl_2,2H_20$, 0.3 g; Tween 80, 2 ml; and 1 ml of a trace metal solution containing $CoCl_2,6H_2O$ (200 mg), $FeSO_4,7H_2O$ (500 mg), $MnSO_4,H_2O$ (160 mg) and $ZnSO_4,7H_2O$ (140 mg), all dissolved in 100 ml of distilled water acidified to pH 3. All but the $CaCl_2$ were dissolved in 1 litre of distilled water and the pH was adjusted to 7.0. The calcium salt was added aseptically after sterilization, to prevent the formation of precipitates. The medium was distributed in 100 ml portions into 500 ml Erlenmeyer flasks, which were inoculated with 5 ml of the TSB culture and incubated for 48 h at 34 °C on a rotary shaker at 250 rev./min.

**Enzyme production.** The mycelium was recovered by centrifugation of the cultures in a Beckman J 21B centrifuge at 11000 *g* for 30 min and washed twice with 5 mM-sodium phosphate buffer, pH 6.0. The mycelium was then resuspended in the original volume of buffer, placed into 500 ml Erlenmeyer flasks and incubated for 18–20 h at 4 °C on the rotary shaker at 250 rev./min. The mixture was centrifuged as above and the supernatant recovered as the crude enzyme preparation. To prevent possible proteolysis, 50 μg of phenylmethanesulphonyl fluoride (BRL, Gaithersburg, MD, U.S.A.)/ml was added.

**Enzyme purification.** Initial purification of the enzyme was carried out by anion-exchange chromatography on a 2.5 cm × 20 cm column packed with 25 g of Accell QMA (Millipore Waters, Milford, MA, U.S.A.); 2.5 l of the crude enzyme preparation was passed through the

Abbreviation used: DNS, dinitrosalicylic acid.

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column at a flow rate of 150 ml/h. The column was then washed with 200 ml of 5 mM-sodium phosphate buffer, pH 6.0. The enzyme was eluted at a flow rate of 100 ml/h with 200 ml of the same buffer containing 100 mM-NaCl. The active fractions were pooled and concentrated to about 15 ml by ultrafiltration in an Amicon cell (Amicon Corp., Danvers, MA, U.S.A.) by using a PM 10 membrane. The solution was washed twice with 20 mM-Tris/HCl buffer, pH 8.5, to lower the salt concentration and to adjust the buffer concentration. Final purification was achieved by h.p.l.c. on a Protein Pak DEAE 5 PW anion-exchange column (Waters Associates) equilibrated with 20 mM-Tris/HCl buffer, pH 8.5, containing 10 mM-NaCl. The proteins were eluted from the column with a linear gradient of 10–500 mM-NaCl in 20 mM-Tris/HCl buffer, pH 8.5. The elution was monitored with a u.v. recorder at 280 nm. The fractions containing the xylanase activity were collected, pooled and concentrated by ultrafiltration. The enzyme was kept at 0 °C. For prolonged storage it was kept at −70 °C.

### Analytical methods

**β-1,4-D-Xylan xylanohydrolase (EC 3.2.1.8).** The assay for β-1,4-D-xylan xylanohydrolase (xylanase) was carried out by incubating at 60 °C for 10 min 1 ml of enzyme solution appropriately diluted in 0.1 M McIlvain buffer, pH 6.0, with 1 ml of an aqueous solution of 1% soluble xylan. The amount of reducing sugars released was determined by the dinitrosalicylic acid (DNS) method of Miller *et al.* (1960), with D-xylose as standard. The blanks consisted of 1 ml xylan solutions incubated like the sample and to which 2 ml of the DNS solution and 1 ml of enzyme were added.

**β-1,4-D-Glucan glucanohydrolase (EC 3.2.1.4).** The activity of β-1,4-D-glucan glucanohydrolase (CM-cellulase) was determined by adding 1.4 ml of 100 mM McIlvain buffer, pH 7.0, and 0.1 ml of enzyme solution to 0.5 ml of a 1% (w/v) aqueous solution of CM-cellulose [type 4M6F, DS (degree of substitution) 0.38–0.55; Hercules Inc., Wilmington, DE, U.S.A.]. The reaction mixture was incubated at 55 °C for 20 min and the amount of reducing sugars produced was determined by the DNS method of Miller *et al.* (1960), with D-glucose as standard. The blanks were prepared in the same manner except that the DNS solution was added before the enzyme. The enzyme activity was also tested directly on Petri plates containing an agar gel in which 1% CM-cellulose (Sigma; medium viscosity, DS 0.65–0.90) was incorporated, by placing the test solutions in wells of 40 μl capacity and incubating overnight at 40 °C. Enzyme activity was detected by Congo Red coloration, by the method of Wood (1980). The sensitivity of this coloration allowed the detection of β-1,4-endoglucanase activity as low as 0.01 μmol/ml.

**β-Xylosidase (EC 3.2.1.37).** The method of analysis for β-xylosidase was carried out as described by Kluepfel & Ishaque (1982), with *p*-nitrophenyl xyloside (Sigma) as substrate, by determining the *p*-nitrophenol liberated by the enzyme action at 40 °C after 30 min.

**Protein.** The protein contents of the supernatants were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

The activities in all enzyme assays were expressed in units, where 1 unit represents 1 μmol of xylose (or glucose, where applicable) released in 1 min by 1 ml of enzyme solution or 1 mg of protein. All results reported are the averages of at least three independent experiments.

**Chromatography of hydrolysis products.** The hydrolysis products generated by time-course digestion with the purified enzyme were analysed by t.l.c. on silica gel G in the solvent system ethyl acetate/acetic acid/water (2:1:1, by vol.) Reducing sugars were detected with aniline phthalate reagent (Partridge, 1949).

**Determination of $M_r$.** The $M_r$ of the purified xylanase was estimated by slab-gel electrophoresis as described by Laemmli (1970). The gels were stained with either Coomassie Blue for proteins or Schiff's reagent for glycoproteins (Glossmann & Neville, 1971).

**Determination of isoelectric point.** Analytical isoelectric-focusing gels were run as described by Vesterberg (1972) in a flat-bed apparatus (FBE.300, Pharmacia Fine Chemicals). The Ampholine range was pH 3.5–10 and the Pharmacia broad-pI kit was used to calibrate the gel. After electrofocusing, the slab gel was cut into two parts, one for staining with Coomassie Blue to localize the endoxylanase, the other for determination of activity. The latter part was cut into 3 mm slices, which were incubated individually for 5 h at 25 °C in 1 ml of sterile reaction mixture containing 1% xylan in McIlvain buffer, pH 6.0. The activity was determined as above.

## RESULTS

Isolation of intact xylanase polypeptide from the culture filtrates of *S. lividans* (mutant 8-7) proved to be difficult, in spite of the high activity (70 units/ml) recorded after 72 h of incubation. The main reason for this was the progressive fragmentation of the enzyme into several active fractions of lower $M_r$. As an alternative method of enzyme production, we investigated the release of enzyme by the mycelium harvested during exponential-phase growth, washed twice with 5 mM-sodium phosphate buffer, pH 6.0, and resuspended in an equal volume of the same sterile buffer, followed by

**Table 1. Purification of xylanase from *Streptomyces lividans* (mutant 8-7)**

| Purification step | Volume (ml) | Protein content (mg/ml) | Activity (μmol/min per ml) | Specific activity (units/mg of protein) | Yield (%) |
|---|---|---|---|---|---|
| Culture supernatant | 4300 | 0.092 | 1.05 | 11.4 | 100 |
| Accell QMA | 850 | 0.039 | 2.50 | 64.1 | 47 |
| H.p.l.c. | 10 | 0.220 | 80.00 | 364.0 | 18 |

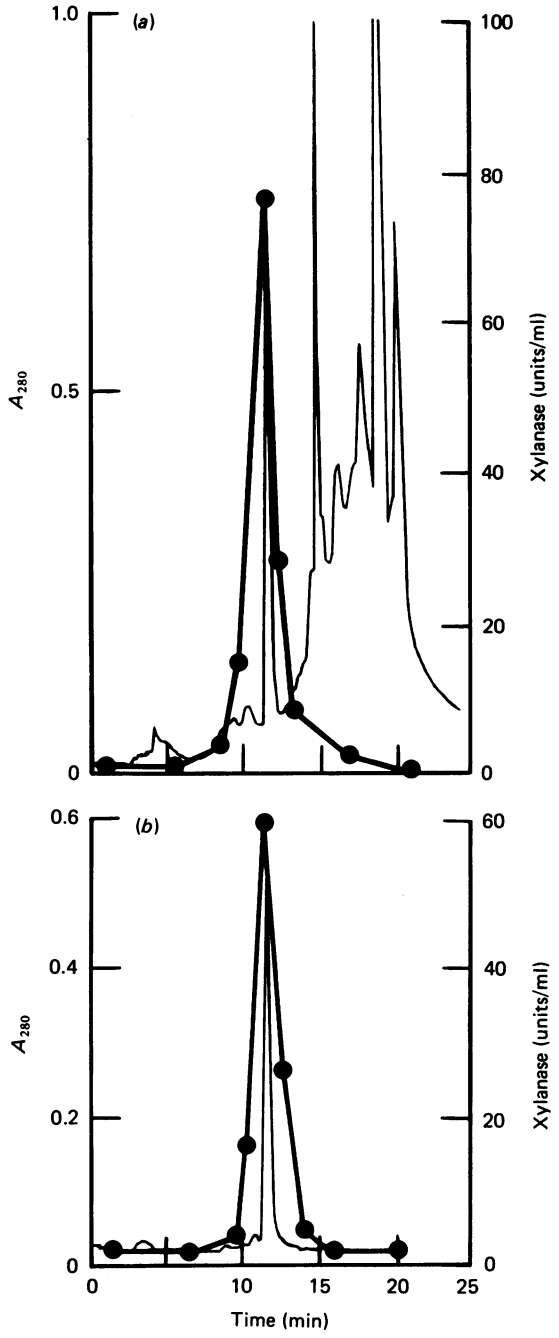


**Fig. 1. Purification of xylanase by h.p.l.c.**

The xylanase was purified on a Protein Pak DEAE 5 PW anion-exchange column under the following conditions: mobile phase, linear ionic-strength gradient of 20 mM-Tris/HCl with 1 M-NaCl, pH 8.5; temperature 20 °C; flow rate 1 ml/min; u.v. detection at 280 nm (——). Assays for enzyme activity were performed as described in the Experimental section (——). The active fraction at 12.6 min in Expt. (*a*) was desalted and rechromatographed in Expt. (*b*).

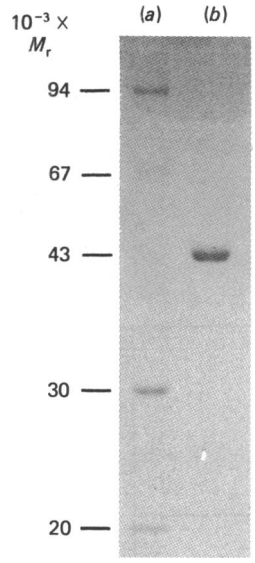


**Fig. 2. SDS/polyacrylamide-gel electrophoresis of xylanase purified by h.p.l.c.**

Track (*a*) contained $M_r$ marker proteins. Phosphorylase ($M_r$ 94000), bovine serum albumin ($M_r$ 67000), ovalbumin ($M_r$ 43000), carbonic anhydrase ($M_r$ 30000) and soya-bean trypsin inhibitor ($M_r$ 20100). Track (*b*) contained the purified xylanase (10 $\mu$g).

incubation for 18 h at 4 °C on a rotary shaker. Under these conditions about 1 unit of xylanase activity/ml was released into the buffer solution. Incubation at 4 °C was preferred to higher temperatures because no degradation of the enzyme preparation occurred, and there was little formation of high-$M_r$ pigment, which interfered with the purification by h.p.l.c. in spite of the pre-purification on an Accell chromatography column. The final purification was achieved on an h.p.l.c. anion-exchange column as shown in Fig. 1. The active fraction was eluted at 12.6 min, corresponding to 300 mM salt concentration. The purification steps are summarized in Table 1. The enzyme was purified 32-fold, with an overall yield of 18%. The specific activity of the pure enzyme was 364 units/mg of protein. No loss of activity was encountered on column chromatography; the decrease in total units recovered was due entirely to the selection of the fractions to be pooled, especially in the first purification step.

Analysis of the purified enzyme by slab-gel electrophoresis revealed one single protein band with an apparent $M_r$ of 43000 calculated from a standard curve as shown in Fig. 2. Analytical isoelectric focusing of the enzyme in a gradient of pH 3.5–10 showed a single protein band with a pI of 5.2. The xylanase activity, as revealed in individual slices of the gel, corresponded exactly to the position of the protein (Fig. 3). The enzyme was not glycosylated, as revealed by negative staining with Schiff reagent (results not shown).

The stability of the xylanase was determined by incubating the enzyme without substrate aseptically in 50 mM McIlvain buffer, pH 6.0, at various temperatures and for different periods of time, after which the activity was determined by the standard assay. The enzyme showed good stability from 0 to 37 °C, whereas above 37 °C the stability decreased rapidly. Fig. 4 shows the stability of xylanase at temperatures of 37–60 °C for 24 h.

The temperature and pH optima for activity were 60 °C and 6.0 respectively (Fig. 5). These optimal values

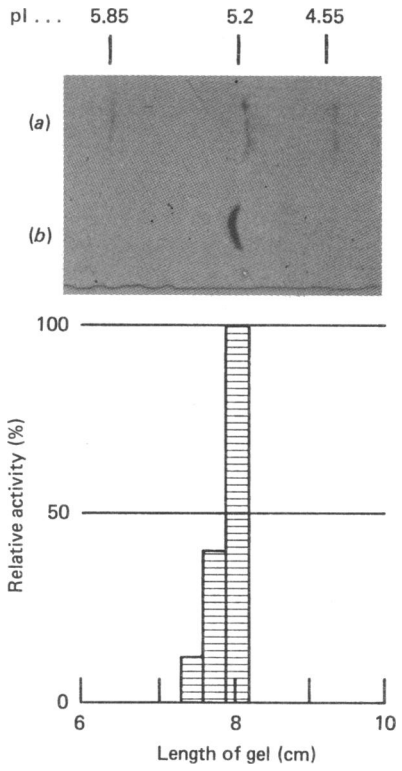


**Fig. 3. Analytical electrofocusing of xylanase purified by h.p.l.c.**

Track (*a*) contained pI marker proteins. These were soya-bean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.20) and bovine carbonic anhydrase B (pI 5.85). Track (*b*) contained the purified xylanase (10 μg). One part of the slab gel was stained with Coomassie Blue (top) and the other part was sliced. Individual slices were assayed for xylanase (bottom).

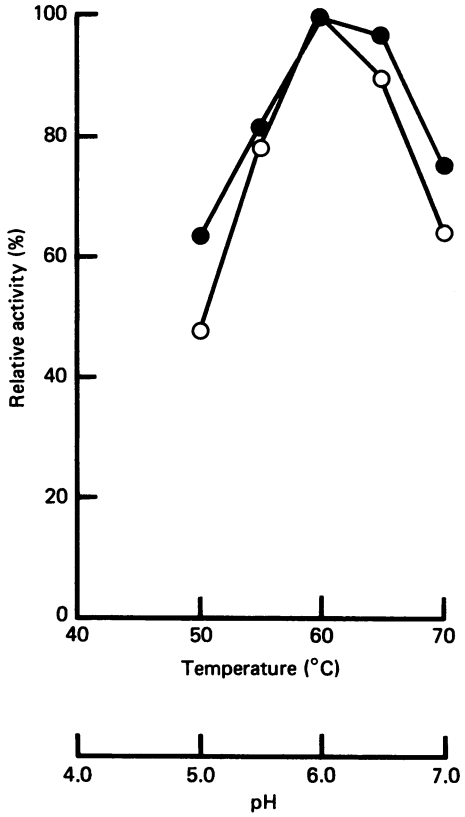


**Fig. 5. Effect of temperature (●) and pH (○) on the xylanase activity**

Purified xylanase (1.5 μg) was incubated for 10 min at the temperatures or pH values indicated, and the activity was measured by the DNS method as described in the Experimental section.

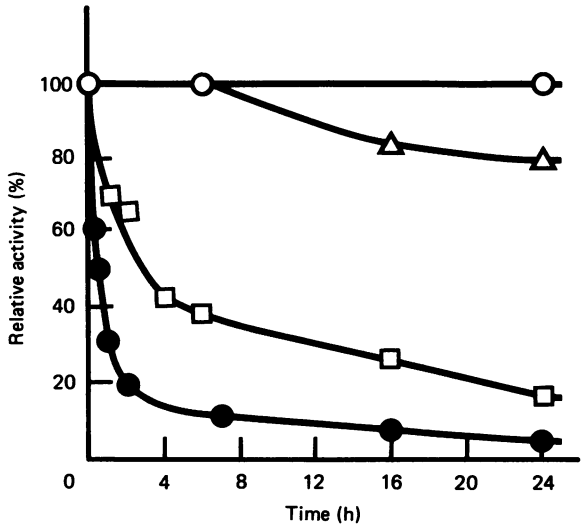


**Fig. 4. Effect of temperature on the xylanase stability**

Purified xylanase at a concentration of 4 μg/ml in 100 mM McIlvain buffer, pH 6.0, was incubated without substrate at 37 °C (○), 43 °C (△), 50 °C (□) and 60 °C (●). The enzyme activity was determined at intervals as described in the Experimental section.

correspond to those previously determined for the crude extracellular xylanase in *S. lividans* 1326 (Kluepfel *et al.*, 1986). The Michaelis–Menten constant was determined at xylan concentrations ranging from 0.1 to 2.0 mg/ml. From a Lineweaver–Burk plot, the $K_m$ was found to be 0.78 mg/ml and the $V_{max.}$ was 0.8 mmol/min per mg of enzyme.

The hydrolysis products of xylan produced by the purified xylanase at different incubation times are shown in Fig. 6. The incubation temperature was 37 °C, chosen for reasons of enzyme stability. Initially, the substrate was hydrolysed to large intermediates, which appeared as a smear on the chromatogram. After 16 h incubation, the end products consisted of a mixture of xylo-oligosaccharides, xylobiose and small amounts of xylose. This indicates that the mode of action of the xylanase is of the endo-type.

The purified enzyme preparation showed no exo- or endo-cellulase activity towards CM-cellulose, as measured by either the reducing-sugar (DNS) method or by the Congo Red coloration on Petri plates. The enzyme was unable to degrade xylobiose, as detected by t.l.c. (results not shown) and was not active on *p*-nitrophenyl β-xyloside; no xylosidase activity therefore was associated with the enzyme preparation.

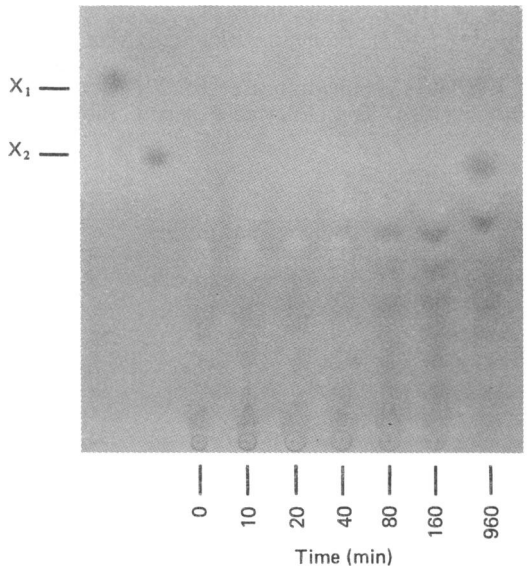


**Fig. 6. T.l.c. of hydrolysis products of xylan by xylanase**

Enzyme (5 $\mu$g) was incubated at 37 °C in a 2 ml solution containing 1% xylan in 100 mM McIlvain buffer, pH 6.0. At the times shown, 25 $\mu$l samples were heated to 100 °C for 10 min to inactivate the enzyme and then spotted on the chromatographic plate. Xylose ($X_1$, 50 $\mu$g) and xylobiose ($X_2$, 50 $\mu$g) were used as standards.

## DISCUSSION

*Streptomyces lividans* produced considerable amounts of extracellular xylanase and to a lesser degree CM-cellulase. The micro-organism also showed a small but significant overall cellulolytic activity (Kluepfel *et al.*, 1986). Preliminary experiments with *S. lividans* 1326 indicated that a cellulase co-purified with the xylanase in the procedure used. The isolation of the xylanase was therefore carried out with the endocellulase-negative mutant 8-7, which facilitated the purification. Washed mycelium, incubated at 4 °C in 5 mM-phosphate buffer, yielded considerably purer xylanase preparations. This procedure limited the binding of the enzyme to its substrate (Paice *et al.*, 1978), thus decreasing the amount of enzyme–substrate complex, which is difficult to purify. In addition, incubation at 4 °C for a short time limited the degradation of the enzyme. Incubations at higher temperatures led to a progressive degradation of the enzyme into smaller molecules, which still retained xylanase activity. Similar observations have been reported for cellulases from *Sporotrichum pulverulentum* (Eriksson & Pettersson, 1982).

The xylanase isolated from *S. lividans* mutant 8-7 resembles that of *Streptomyces* sp. KT-23 in several respects (Nakajima *et al.*, 1984). The $M_r$ as well as the pH and temperature optima are almost the same. The pI, however, is different, 5.2 instead of 6.9 for strain KT-23. The enzyme shows a lower affinity for xylan, 0.78 mg/ml instead of 0.2 mg/ml reported for the other enzyme. The major difference observed between the two enzymes is the mode of hydrolysis of xylan. Our enzyme degraded xylan progressively by random cuts of the molecules, producing first large xylo-oligosaccharide fragments and, after complete digestion, a mixture of lower xylo-oligosaccharides, large amounts of xylobiose, and very small amounts of xylose. Comparing these results with those obtained from xylanases of *Streptomyces* sp. KT-23 (Nakajima *et al.*, 1984), the latter produce a mixture of xylobiose and xylotriose from the beginning and, after complete digestion, mainly xylobiose with trace amounts of xylose.

The $K_m$ value (0.78 mg/ml) of *S. lividans* xylanase is low compared with those of fungal and yeast xylanases, which range from 4 to 20 mg of xylan/ml (Woodward, 1984).

The xylanolytic and cellulolytic enzyme systems in some filamentous fungi are likely to be under separate regulatory control (Biely, 1985). When grown on xylan, such species produce specific xylanases, with little or no cellulases. When grown on cellulose, however, cellulases are produced together with xylanases. In *S. lividans* (Kluepfel *et al.*, 1986) and in *S. flavogriseus* (Kluepfel & Ishaque, 1982) the opposite situation was observed: mycelium from a cellulose medium produces only residual amounts of xylanase, yet when grown on xylan both the xylanase and the cellulase enzyme systems are strongly induced. These observations were confirmed to some extent by mutagenesis experiments on the *S. lividans* wild strain. It was possible to isolate cellulase-negative mutants and cellulase- and xylanase-negative double mutants, but so far we have been unable to obtain xylanase-negative mutants (D. Kluepfel, unpublished work). This seems to indicate that parts of the xylanase gene exert some control on the expression of the cellulase gene. Additional genetic and biochemical studies are needed to clarify the regulation process of xylanase and cellulases in *S. lividans*.

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